

SPECIFICATION

Chimeric proteins, their heterodimer complexes, and platelet substitutes

Technical Field:

Sub D1 The present invention relates to chimeric proteins consisting of an integrin and an immunoglobulin, their heterodimer complexes, a production process thereof, their applications as drugs and reagents, etc. Furthermore, the present invention relates to medicinal application of isolated extracellular matrix receptors such as integrin-immunoglobulin chimeric protein heterodimer complexes, as platelet substitutes.

Background Arts:

Various cells have receptors which mediate the adhesion between a cell and a cell and receptors which mediate the adhesion between a cell and an extracellular matrix, and these receptors play important roles in immune reaction, inflammatory reaction, development, morphogenesis, wound healing, hemostasis, cancerous metastasis, etc. By separating and identifying the receptors which participate in these phenomena, the existence of so-called cell adhesion molecules has been clarified. Many of the molecules identified one after another are classified in reference to their structural features into integrin superfamily, immunoglobulin superfamily, selectin family, cadherin family, etc. (Corliss,

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T. M. and Harlan, J. M., Blood, 84, 2068-2101 (1994)). Of these families, the immunoglobulin superfamily, selectin family and cadherin family mediate mainly the adhesion between a cell and a cell, while the integrin superfamily is the so-called extracellular matrix receptors which mediate the adhesion to extracellular matrices such as fibronectin and collagens. In addition, extracellular matrix receptors which do not belong to any of these adhesion molecule families include CD26 (DDP1V), CD44, GPIV, GPVI, GPIb-vWF, etc. CD26 is a receptor for collagens, and CD44 is a receptor for hyaluronic acid, fibronectin and collagens ("Adhesion Molecules" p. 32-42, Masayuki Miyasaka (1991), Medical View (in Japanese)). Furthermore, it is reported that among the membrane glycoproteins (GPs) existing on platelets, GPIV, GPVI, GPIb-vWF, etc. are also collagen receptors ("Platelet Receptors", p. 119-132, Minoru Ohkuma et al., (1992), Kinpodo (in Japanese)).

Sub D2 A receptor belonging to the integrin superfamily has a heterodimer complex structure in which two subunits, α -chain and β -chain as mutually different membrane proteins are associated with each other non-covalently (Hynes, R. O., Cell, 48, 549-554 (1987)). In the past, the integrin superfamily was classified into three subfamilies; β 1 integrin, β 2 integrin and β 3 integrin. Later, new β chains and α chains were discovered one after another, and presently eight β

chains ($\beta 1, \beta 2, \beta 3, \beta 4, \beta 5, \beta 6, \beta 7$ and $\beta 8$ and fifteen α chains ($\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 9, \alpha v, \alpha L, \alpha M, \alpha X, \alpha 11b$ and αE) have been identified (Elner, S. G. and Elner, V. M. Inv. Ophthal. Vis. Sci., 37, 696-701 (1996)). It is known that each β chain is associated with one to eight α chains, and as a result, 21 pairs of an α chain and a β chain, i.e., integrin molecules have been identified (Elner, S. G. and Elner, V. M., Inv. Ophthal, Vis. Sci., 37, 696-701 (1996)). They include $\alpha 4 \beta 1$ (VLA-4, $\beta 1$ integrin), $\alpha L \beta 2$ (LFA-1, $\beta 2$ integrin), $\alpha M \beta 2$ (Mac-1, $\beta 2$ integrin), $\alpha 11b \beta 3$ (GP11b/IIIa, $\beta 3$ integrin), etc. now targeted for drug development (Drug and Market Development, 6, 201-205 (1995)). Many other integrins are also expected to have relations with diseases.

Sub-D3 The heterodimer complex structure of an integrin plays an important role in binding to a ligand (Hynes, R. O., Cell, 48, 549-554 (1987)). For example, it is estimated that the ligand binding region on an integrin consists of both an α chain and a β chain (Hynes, R. O., Cell, 69, 11-25 (1992)). The fact that integrins having the same α chain but associated with a different β chain, or integrins having the same β chain but associated with a different α chain are respectively different in substrate specificity (Elner, S. G. and Elner, V. M., Inv. Ophthal, Vis. Sci. 37, 696-701 (1996)) supports this assumption. On the other hand, it was reported that the α chains of some integrins have an sequence called an I domain

consisting of about 180 amino acids inserted in the molecule, and data suggest that the I domain only could be bound to a ligand were reported (Ueda, T. et al., Proc. Natl. Acad. Sci. USA, 91, 10680-10684 (1994)). However, it was also reported that the I domain of an α domain and the integrin as its original heterodimer complex are different in the style of binding to a ligand (Kamata, T. and Takada, Y., J. Biol. Chem., 269, 26006-26010 (1994)). It is also not clarified yet whether such parameters as specificity and affinity to a ligand are identical. It is not reported that in the case of an integrin not containing the I domain, for example, in the case of $\alpha 4 \beta 1$ a partial structure only is bound to a ligand.

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Sub D4 If any integrin isolated and prepared retains its heterodimer complex structure, hence the ligand binding capability, it can be used for studying the style of binding to a ligand in a state close to nature. Furthermore, it can be used as it is as a drug and can also be used as a reagent for measuring the amount of a ligand in tissue or serum or as a material for searching for adhesion inhibiting compounds very usefully. However, isolating and preparing an integrin with its function retained is said to be very difficult. One reason is that since the association between an α chain and a β chain of an integrin is maintained non-covalently as described before, they are easily dissociated during isolation and preparation. Since an integrin is a membrane protein, the

necessity of using a surfactant, etc. for solubilization is considered to be a large cause in the dissociation of the complex. In other words, the non-covalent preservation of functional structure inhibits the preparation of such an integrin.

Sub D5 In spite of the difficulty as described above, some cases were reported, in which an integrin heterodimer complex was isolated and prepared with its function retained. For cases of $\alpha 2 \beta 1$, $\alpha 5 \beta 1$ and $\alpha v \beta 3$, it was reported that the binding to a ligand can be determined by letting a liposome incorporate an integrin purified by using affinity column chromatography (Santoro, S. A. et al., Biochem. Biophys. Res. Comm., 153, 217-223 (1988), Pytela, R. et al. Cell, 40, 191-198 (1985), Pytela, R. et al., Method Enzymol., 144, 475-489 (1987)). For other cases, it was that if purified $\alpha 5 \beta 1$ or $\alpha v \beta 3$ is coated on a plate, a peptide which inhibits the cell adhesion through the integrin can be selected (Koivunen, E. et al., J. Biol. Chem., 268, 20205-20210 (1993), Healy, J. M. et al., Biochemistry, 34, 3948-3955 (1995)). For further other cases, it was reported that if purified $\alpha v \beta 3$ or $\alpha 4 \beta 1$ is coated on a plate, the binding to a ligand can be determined (Charo, I. F. et al., J. Cell Biol., 111, 2795-2800 (1990), Makarewicz, R. et al., J. Biol. Chem., 269, 4005-4011 (1994), Paul Mould, A. et al., J. Biol. Chem., 269, 27224-27230 (1994)). For a still further other case, it was reported that if an extracellular

portion of $\alpha 11b\beta 3$ heterodimer complex prepared by gene manipulation is coated on a plate through a complex specific antibody, the binding to a ligand can be determined (Gulino, D. et al., Eur. J. Biochem., 227, 108-115 (1995)). These cases suggest that to exert the function of a purified integrin, its heterodimer complex must be bound to or included in any carrier. The reason why a carrier is considered to be necessary is that since a heterodimer complex is associated non-covalently in a solution, it tends to be dissociated and as a result, cannot retain its functional structure. In the finally stated case, only a molecule with a heterodimer complex structure is selected using a complex specific antibody, in a design to determine the binding in a state where both the chains are not dissociated from each other.

See DG → As a case requiring no carrier, it was reported that purified $\alpha 1\beta 1$, or $\alpha 2\beta 1$ allows the determination of the bonding to a ligand dependent on high concentration of metal ions even without using any carrier (Pfaff, M. et al., Eur. J. Biochem., 225, 975-984 (1994)). In this case, the surfactant added in the process of purification plays a role similar to that of a liposome, acting as a carrier. For a further other case, it was reported that an extracellular of $\alpha M\beta 2$ heterodimer complex prepared by using gene manipulation is bound to a ligand (Berman, P. W. et al., J. Cell Biochem., 52, 183-195 (1993)). These cases do not suggest the necessity of

any carrier as described before, but the disadvantage that the association of molecules in a heterodimer complex is retained non-covalently is not improved.

As a still further other case, a chimeric protein consisting of α d and an immunoglobulin is disclosed (Japanese Patent Laid-Open (Kokai) No. 8-507933), but only the result of immune precipitation is reported, without examining the binding to a ligand. Furthermore, since a β chain is not expressed in the chimeric protein as an immunoglobulin, the binding between an α chain and a β chain remains non-covalent.

The above facts suggest that any integrin with an α chain and a β chain structurally stably associated and with its function retained has never been successfully prepared. That a complex structure is unstable restricts the use of its molecule.

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SuD9 Of the molecules belonging to the integrin superfamily, integrin $\alpha 2 \beta 1$ is an extracellular matrix receptor found to be expressed in T cells, platelets, etc. activated for long time. However, it was reported that the $\alpha 2 \beta 1$ on the cell surfaces of platelets and fibroblasts is bound to collagens only and that the $\alpha 2 \beta 1$ on the surfaces of vascular endothelial cells is bound to both collagens and laminins (Elices, M. J. et al., Proc. Natl. Acad. Sci. USA, 86, 9906-9910 (1989)), and it is speculated that the function of $\alpha 2 \beta 1$ becomes different,

depending on cells.

Sub 8 > In relation to the conditions of diseases, there are reports to suggest that integrin $\alpha 2 \beta 1$ plays an important role for wound healing and cancerous metastasis (Shiro, J. A. et al., Cell, 67, 403-410 (1991), Chen, F. et al., J. Exp. Med., 173, 1111-1119 (1991), Chan, B. M. C. et al., Science, 251, 1600-1602 (1991)). Furthermore, it was reported that from the analysis of platelet function of patients with bleeding tendency, the adherence of platelets and collagens through integrin $\alpha 2 \beta 1$ has close relation with the first step of hemostasis and thrombosis process (Nieuwenhuis, H. K. et al., Nature, 318, 470-472 (1985)). Though the relations of integrin $\alpha 2 \beta 1$ with conditions of diseases are suggested like this, any medical application of using the integrin $\alpha 2 \beta 1$ protein and other isolated extracellular matrix receptor proteins under physiological ion condition or in the presence of plasma components has not been examined.

On the other hand, the necessity for artificial substitutes of platelets used as blood preparations in the clinical field is growing, and various attempts have been reported (Progress of Medicine 179, 406-407 (1996), Clinical Blood 37, 1353-1361 (1997) (respectively in Japanese)). However, they are not yet practically available.

Disclosure of the Invention:

Sub 89 > The present invention relates to chimeric proteins in

which the α chain and β chain of an integrin are combined with the heavy chain or light chain of an immunoglobulin, their heterodimer complexes, a production process thereof, a method for testing the binding of an integrin-immunoglobulin chimeric protein heterodimer complex to a ligand and a cell, substances bound to an integrin obtained by using the method, a method for searching for a substance inhibiting the binding between an integrin and a ligand using the integrin-immunoglobulin chimeric protein heterodimer complex, substances for inhibiting the binding, and the application of integrin-immunoglobulin chimeric protein heterodimer complexes as drugs and reagents. Furthermore, the present invention relates to platelet substitutes containing an integrin-immunoglobulin chimeric protein heterodimer complex or any other isolated extracellular matrix receptor as an active ingredient.

Brief Description of the Drawings:

Fig. 1 shows that $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex is bound to VCAM-1 expressing cell, and that the binding is inhibited by an anti-integrin antibody or EDTA, a cationic chelating agent.

Fig. 2 shows that $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex is bound to CS-1 peptide, and that the binding is inhibited by an anti-integrin antibody or EDTA, a cationic chelating agent.

Fig. 3 shows that the binding between $\alpha 4 \cdot \text{IgG}$ heavy

chain- β 1IgG heavy chain chimeric protein heterodimer complex and CS-1 peptide is inhibited by GPEILDVPST, and is not inhibited by any other peptide.

Fig. 4 shows that α 2-IgG heavy chain- β 1IgG heavy chain chimeric protein heterodimer complex is bound to a collagen, and that the binding is inhibited by an anti-integrin antibody and EDTA, a cationic chelating agent.

Fig. 5 shows that α 2-IgG heavy chain- β 1IgG heavy chain chimeric protein heterodimer complex liposome is bound to a collagen in the presence of plasma.

Fig. 6 shows that the binding of α 2-IgG heavy chain- β 1IgG heavy chain chimeric protein heterodimer complex liposome to a collagen is inhibited by an anti-integrin antibody or EDTA, a cationic chelating agent.

The Best Embodiments of the Invention:

The extracellular matrix receptors in the present invention refer generally to the receptors which mediate the adhesion between a cell and an extracellular matrix. The receptors include the integrin superfamily having a heterodimer complex structure in which an α chain and a β chain are non-covalently associated with each other as two membrane proteins (Corliss, T. M. and Harlan, J. M. Blood, 84, 2068-2101 (1994)), and other receptors such as CD26 (DDPIV), CD44, GPIV, GPVI, GPb-vWF, etc. The integrins in the present invention refer to molecules belonging to the integrin

superfamily, and also include the isomers of the molecules belonging to the family. The α chains of the present invention include 15 α chains, i.e., $\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 9, \alpha v, \alpha L, \alpha M, \alpha X, \alpha 11b$ and αE , and among them, $\alpha 4$ and $\alpha 2$ are preferable, though preferable α chains are not limited to them. The β chains of the present invention include eight β chains, i.e., $\beta 1, \beta 2, \beta 3, \beta 4, \beta 5, \beta 6, \beta 7$ and $\beta 8$ and among them, $\beta 1$ is preferable, though preferable β chains are not limited to it. The integrin molecules as pairs respectively consisting of an α chain and a β chain include the twenty one integrins stated in Elner, S. G. and Elner, V. M., Inv. Ophtal. Vis. Sci., 37, 696-701 (1996), though not limited to them.

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A chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin refers to a molecule in which the extracellular region of the α chain of an integrin is bound to the constant region of the heavy chain or light chain contained an immunoglobulin. In this case, a chimeric protein in which N terminus side of the protein is integrin molecule and then connected to an immunoglobulin molecule side by side is preferable. A chimeric protein consisting of the β chain of an integrin and the heavy chain or light chain of an immunoglobulin refers to a molecule in which the extracellular region of the β chain of an integrin is bound to the constant

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region of the heavy chain or light chain contained in an immunoglobulin. Also in this case, a chimeric protein in which N terminus side of the protein is an integrin molecule and then connected to an immunoglobulin molecule side by side is preferable. In either case of α chain or β chain, a chimeric protein bound to the heavy chain of an immunoglobulin is preferable.

The isotype of the immunoglobulin to be bound to the α chain or β chain is not especially limited. Any of IgG, IgM, IgA and IgE can be used, but it is preferable to use IgG. The subclasses of IgG include IgG₁, IgG₂, IgG₃ and IgG₄, but it is preferable to use IgG₁. Furthermore, it is possible to use a molecule with a dimer structure having a disulfide bond between molecules instead of the immunoglobulin.

Sub D12 In the present invention, a molecule in which a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of the β chain of the integrin and the heavy chain or light chain of the immunoglobulin are associated with each other is called an integrin-immunoglobulin chimeric protein heterodimer complex. In this case, a combination consisting of α chain-immunoglobulin heavy chain (which means a chimeric protein consisting of an α chain and the heavy chain of an immunoglobulin; hereinafter this applies) and β chain-immunoglobulin heavy chain, a combination consisting of

α chain-immunoglobulin heavy chain and β chain-immunoglobulin light chain, and a combination consisting of α chain-immunoglobulin light chain and β chain-immunoglobulin heavy chain are preferable. A combination consisting of α chain-immunoglobulin heavy chain and β chain-immunoglobulin heavy chain is more preferable.

Sub D13 In the integrin-immunoglobulin chimeric protein heterodimer complex of the present invention, the α chain can be $\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 9, \alpha v, \alpha L, \alpha M, \alpha X, \alpha 11b$ or αE , and the β chain can be $\beta 1, \beta 2, \beta 3, \beta 4, \beta 5, \beta 6, \beta 7$ or $\beta 8$. It is preferable that the α chain is $\alpha 4$ or $\alpha 2$ and that the β chain is $\beta 1$, though preferable chains are not limited to them.

Sub D14 The process for preparing an integrin-immunoglobulin chimeric protein heterodimer complex is described below, but the process is not limited thereto.

Sub D15 A DNA coding for the α chain and β chain of an integrin can be obtained using the information of known cDNA sequences by such a method as gene amplification based on the PCR method, cDNA cloning or DNA synthesis. For example, the DNA sequences of $\alpha 4$ and $\beta 1$ are already reported in literature (Takada, Y. et al., EMBO J., 8, 1361-1368 (1989), Scott Argraves, W. et al., J. Cell Biol., 105, 1183-1190 (1987)). A DNA coding for the α chain and β chain of an integrin can also be obtained by the expression cloning using an antibody, etc. For binding to a DNA coding for the constant region of

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an immunoglobulin, it is desirable to take out a DNA coding for the extracellular portions only of the α chain and β chain of an integrin. For this purpose, it is preferable to use the PCR method and DNA synthesis. The extracellular portion of either an α chain or β chain refers to the polypeptide sequence on the N terminus side from the portion speculated to be the transmembrane portion. Its partial sequence can also be used as far as the ligand binding capability is retained, but it is preferable to use most of the portion considered to be an extracellular region. For taking out a DNA, it is necessary to adjust for adaptation of frames after linking to a DNA coding for an immunoglobulin. For example, this can be achieved by modifying the primer when a DNA fragment is taken out by the PCR method. In this case, it is desirable to design for ensuring that amino acid modification is not caused by the base substitution of the primer. However, amino acid substitution is allowed as far as the function of the chimeric protein is not changed. For obtaining a DNA by chemical synthesis, the purpose can be achieved by designing a sequence to ensure the linking to a DNA coding for an immunoglobulin. In the case of cDNA, a DNA capable of being bound to a DNA coding for an immunoglobulin can be prepared by using DNA fragmentation and a synthetic DNA.

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Then, a DNA coding for an immunoglobulin is prepared. In

the present invention, it is desirable to use DNAs coding for the heavy chain and light chain of a human immunoglobulin, but DNAs coding for an immunoglobulin of another animal species can also be used. The preparation of a DNA coding for human IgG is already reported (Ellison, J. W. et al., Nucleic Acids Res., 10, 4071-4079 (1982)), but the preparation is not limited to this method. Any method similar to the above mentioned method for preparing DNAs coding for the α chain and β chain of an integrin can also be used. In the present invention, for the heavy chain of a human immunoglobulin, it is preferable to use a genomic DNA, but a cDNA can also be used. As the DNA for the heavy chain of a human immunoglobulin, it is preferable to use a portion coding for the hinge region, CH2 region or CH3 region, but a DNA coding for the entire constant region of CH1 - CH3 can also be used. For the light chain of an immunoglobulin, a DNA coding for the CL region is used. Finally, a DNA coding for the extracellular portion of an α chain or β chain and a DNA coding for the constant region of human immunoglobulin heavy chain are linked with in frame. The obtained DNA codes for a polypeptide starting from the methionine of translation initiation and having the signal sequence of the α chain or β chain of an integrin, its extracellular region and the constant region of human immunoglobulin heavy chain linked in this order.

Sub D17 The DNA coding for a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin, or the DNA coding for a chimeric protein consisting of the β chain of an integrin and the heavy chain or light chain of an immunoglobulin respectively obtained in the above is functionally linked in a proper expression control sequence, to obtain a recombinant vector. The general methods concerning gene recombination such as the method for preparing the recombinant vector, the method for transfecting it into a cell are described in a published book ("Molecular Cloning", Sambrook et al., (1989) Cold Spring Harbor Lab. Press, New York), but the methods are not limited to those stated there. In the present invention, it is desirable to use an expression control sequence suitable for protein expression in animal cells. For example, for manifestation of insect cells, polyhedrin promotor, p10 promotor, etc. are generally used as expression control sequences, and for expression of other animals' cells, SR α promotor, cytomegalovirus derived promotor, simian virus 40 derived promotor, polyhedrin promotor, p10 promotor, etc. are used. However, the expression control sequences are not limited to them. In the present invention, it is preferable to use SR α promotor.

Sub D18 If the obtained recombinant vector is transfectd into a cell, a cell capable of producing an integrin-immunoglobulin

chimeric protein heterodimer complex can be obtained. In this case it is preferable to use an animal derived cell as a host. For example, COS cell (simian renal cell), CHO cell (Chinese Hamster ovarian cell), Sf9 (insect cell), etc. are generally used as hosts. Furthermore, myeloma cells such as P3U1 and Y3 can also be used. Other established cell lines and cloned cells can also be used, but the cells used as hosts are not limited to them. In the present invention, it is preferable to use a CHO cell.

It is known that the methods for transfecting a recombinant vector into a cell include the lipofectin method, calcium phosphate method, electroporation method, etc., and any of the methods can be used. The method is not limited to them. It is preferable that when a cell is transfected by using a recombinant vector, a recombinant vector for expression of a chimeric protein consisting of the α chain of an integrin and the heavy chain or light chain of an immunoglobulin and a recombinant vector for expression of a chimeric protein consisting of the β chain of the integrin and the heavy chain or light chain of the immunoglobulin are transfected into the cell one after another using different drug resistance markers. The recombinant vectors can be transfected in any order or simultaneously. It is desirable that the two recombinant vectors to be transfected are vectors for expression of a combination consisting of α chain.

immunoglobulin heavy chain (which means a chimeric protein consisting of an α chain and the heavy chain of an immunoglobulin; hereinafter this applies) and β chain · immunoglobulin heavy chain, or α chain · immunoglobulin heavy chain and β chain · immunoglobulin light chain, or α chain · immunoglobulin light chain and β chain · immunoglobulin heavy chain. Any of these combinations can be adopted, but a combination of recombinant vectors for expression of α chain · immunoglobulin heavy chain and β chain · immunoglobulin heavy chain is desirable.

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F-4 In any transfection method and any combination of vectors, it is important to select a cell which is transfected by the two recombinant vectors and produces a chimera protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimera protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin simultaneously almost by the same amounts. This can be achieved by measuring the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin produced in the cultured supernatant solution of the cell transfected by the recombinant vectors. For measurement, for example, the transfected cell can be cultured in a medium containing ^{35}S according to any publicly known method, for

labeling the proteins, and the amounts of the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin existing in the cultured supernatant solution can be estimated by immunoprecipitation using an anti- α chain antibody or an anti- β chain antibody respectively. As another method, the amounts the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin existing in the cultured supernatant solution can be estimated according to the ELISA method using an anti-human immunoglobulin antibody and an anti- α chain antibody or an anti- β chain antibody. Anyway, it is preferable to select a clone which produces almost the same large amounts of the chimeric proteins of the α and β chains in the culture supernatant, for preparing an integrin-immunoglobulin chimeric protein heterodimer complex. The methods for labeling proteins, the methods of immunoprecipitation and the general methods of ELISA are described in a published book ("Antibody" Harlow, E., and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York), but the methods are not limited to them. Any other method can also be used for detecting chimeric proteins.

The obtained transfected cell can be cultured according to

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a general cell culture method, to produce an integrin-immunoglobulin chimeric protein heterodimer complex. It is preferable that the medium contains about 5% of serum of a low immunoglobulin concentration, but any generally known serum-containing medium or a serum-less medium can also be used. After completion of cell culture, the cells and solid matter are removed by such operation as centrifugation, and the culture supernatant containing an integrin-immunoglobulin chimeric protein heterodimer complex is collected.

It can be estimated that the cultured supernatant solution contains not only the integrin-immunoglobulin chimeric proteins in which the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin form a heterodimer complex, but also the chimeric protein consisting of an α chain and the heavy chain or light chain of an immunoglobulin and a chimeric protein consisting of a β chain and the heavy chain or light chain of an immunoglobulin which do not form the heterodimer complex. However, since the molecules other than the heterodimer complex cannot be bound to a ligand, the supernatant solution can be used as a reagent for testing the binding to a ligand or cell, or searching for a substance inhibiting the binding between an integrin and a ligand, or for searching for a substance capable of being bound to an

integrin, or for measuring the ligand amount of an integrin. These methods of utilization are basically the same as those for using a purified integrin-immunoglobulin chimeric protein heterodimer complex described later.

14/5 ~~An integrin-immunoglobulin chimeric protein heterodimer complex can be purified by an established method using a protein A column chromatography by use of the nature of the immunoglobulin portion. Furthermore, affinity chromatography using an antibody against the α or β chain can also be used. Moreover, the purification can also be achieved by affinity chromatography with a ligand bound to a carrier. General chromatographic methods can also be used in combination for the purification. If publicly known cases in which integrin molecules are purified by these methods (Pytela, R. et al., Methods Enzymol., 144, 475-489 (1987), Santoro, S. A. et al., Biochem. Biophys. Res. Comm., 153, 217-223 (1988), Charo, I.F. et al., J. Cell Biol., 111, 2795-2800 (1990), Makarem, R. et al., J. Biol. Chem., 269, 4005-4011 (1994), Pfaff, M. et al., Eur. J. Immunol., 225, 975-984 (1994), Gulino, D. et al., Eur. J. Biochem., 227, 108-115 (1995), etc.) are applied, the purification of an integrin-immunoglobulin chimeric protein heterodimer complex can be achieved.~~

Jul 820 ~~A purified integrin-immunoglobulin chimeric protein heterodimer complex can be identified as a protein showing at least one band under non-reducing condition and at least two~~

bands under reducing condition by SDS-PAGE. It can also be confirmed from it, that the heterodimer is linked by the disulfide bond between immunoglobulin heavy chains. It sometimes occurs that a plurality of bands are detected under reduction, but this is considered to be probably because intramolecular cleavage of the α chain has occurred. Especially, with $\alpha 4$, this phenomenon is known (Hemler, M.E. et al., J. Biol. Chem., 262, 11478-11485 (1987)). Furthermore, it can be confirmed by the Western blotting method that the respective bands indicate chimeric proteins. As another method, it can be confirmed by said ELISA method combining an anti- α chain antibody, anti- β chain antibody and anti-human immunoglobulin antibody, that the obtained molecule is an integrin-immunoglobulin chimeric protein heterodimer complex. That is, the molecule can be identified as a protein molecule with epitopes for all the antibodies. As a further other method, an integrin-immunoglobulin chimeric protein heterodimer complex can also be identified by immunoprecipitation. In this case, if the purified protein is labeled by ^{35}S , or ^{125}I or biotin, etc. according to any known method, and immunoprecipitated using an anti- α chain antibody, anti- β chain antibody and anti-human immunoglobulin antibody, the same electrophoretic pattern can be obtained in every case. So, it can be confirmed that the integrin-immunoglobulin chimeric protein heterodimer complex has the

intended structure. Furthermore, even if a condition to dissociate the integrin complex on a cell membrane such as the coexistence of EDTA or boiling in the presence of SDS is applied, the immunoprecipitation pattern is not changed. So, it can be confirmed that the obtained integrin-immunoglobulin chimeric protein heterodimer complex is a structurally stabilized complex. The methods for confirming an integrin-immunoglobulin chimeric protein heterodimer complex are not limited to those stated above.

The binding between a prepared integrin-immunoglobulin chimeric protein heterodimer complex and a ligand can be tested as described below. After a ligand and an integrin-immunoglobulin chimeric protein heterodimer complex are brought into contact with each other, to make a mixture, the amount of the integrin-immunoglobulin chimeric protein heterodimer complex bound to the ligand or the amount of the ligand bound to the integrin-immunoglobulin chimeric protein heterodimer complex is measured. The amount of an integrin-immunoglobulin chimeric protein heterodimer complex can be measured by labeling the complex itself by a fluorescent dye or enzyme or radioisotope, etc. The amount of a ligand can also be measured by any similar method. A detection method such as SPA (Amasham) can also be used for the measurement. Furthermore, any reagent which can recognize a complex or ligand labeled by a fluorescent dye, enzyme or radioisotope,

etc. can also be used for the measurement. The reagent for recognizing an integrin-immunoglobulin chimeric protein heterodimer complex can, for example, be an anti-human immunoglobulin antibody. In this test, it is preferable to bind the molecule to be detected, to any carrier such as a bead or plate. As a ligand, its entire molecule can be used, but a portion retaining the binding activity to an integrin can also be taken out for use. For example, for integrin $\alpha 4 \beta 1$ or integrin $\alpha 2 \beta 1$, its ligand, fibronectin or collagen or its peptide fragment bound to a carrier can also be used.

Methods similar to the above can be used to test the binding between an integrin-immunoglobulin chimeric protein heterodimer complex and cells. The amount of the cells bound to a complex can be measured by labeling the cells by a fluorescent dye or radioisotope or using a reagent reacting with the cells, for example, an antibody reacting with a surface antigen. If something like a tissue section is used instead of cells, the amount of the bound integrin-immunoglobulin chimeric protein heterodimer complex is measured by any of the above mentioned methods.

The methods for examining the binding between an integrin-immunoglobulin chimeric protein heterodimer complex and a ligand or cell described above can be used for obtaining a substance inhibiting the binding between an integrin and a ligand, for example, for obtaining an antibody, polypeptide,

peptide or low molecular weight compound. It is preferable to premix a sample and an integrin-immunoglobulin chimeric protein heterodimer complex, and then to measure the amount of the integrin-immunoglobulin chimeric protein heterodimer complex bound to a ligand in any of the above mentioned measuring systems. If the amount of the bound integrin-immunoglobulin chimeric protein heterodimer complex is lowered by adding a certain sample, it can be judged that the sample has inhibitory activity. However, in this system, a substance with metal ion chelating action or a substance with surfactant action, etc. may give a false positive result. The sources of samples used include the following integrin bound substances, peptide fragments of ligands, their derivatives, marketed compounds, etc., but are not limited to them.

Sub 022 A case where a purified integrin was coated on a plate to search for a peptide to be bound was reported (Healy, J. M. et al., Biochemistry 34, 3948-3955 (1995)). Even if the integrin-immunoglobulin chimeric protein heterodimer complex obtained in the present invention is used, a substance to be bound to an integrin can be similarly searched for. Especially when the chimeric protein heterodimer complex of the present invention is used, the operation to remove the non-specifically bound substances can be effected under more severe conditions. So, the operation can be simplified advantageously. Furthermore, since the complex is not

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dissociated during operation, a bound substance can be selected more specifically advantageously. Known sources suitable for selecting bound substances include a phage peptide library (e.g., Scott, J. K. and Smith, G. P., Science, 249, 386-390 (1990)) and a DNA oligomer library (e.g., O'Connel, D. et al., Proc. Natl. Acad. Sci. USA, 93, 5883-5887 (1996), but in the present invention, it is preferable to use the former.

Furthermore, the method of testing the binding between an integrin-immunoglobulin chimeric protein heterodimer complex and a ligand or cell can also be used as a method for measuring the amount of an integrin ligand in a body fluid or tissue.

Moreover, the integrin-immunoglobulin chimeric protein heterodimer complexes of the present invention can also be used as drugs. The present invention has clarified that integrins and other isolated extracellular matrix receptors can be used as platelet substitutes.

Sub D23 An extracellular matrix receptor preferably used as a platelet substitute is an integrin. The α chain of the integrin can be $\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 9, \alpha v, \alpha L, \alpha M, \alpha X, \alpha 11b$ or αE , and among them, $\alpha 2$ is preferable. The β chain can be $\beta 1, \beta 2, \beta 3, \beta 4, \beta 5, \beta 6, \beta 7$ or $\beta 8$, and among them, $\beta 1$ is preferable. Integrin $\alpha 2 \beta 1$ is more preferable. The receptor source for isolation can be a tissue or cell expressing an

extracellular matrix receptor, or a dissolved membrane fraction of a receptor expressing cell prepared by gene manipulation, etc. It is more preferable to design for obtaining a soluble protein by modifying a receptor gene by gene recombination, and to use the cultured supernatant solution of the cells capable of producing it, as a source. Furthermore in the design of the soluble protein, it is preferable that the functional structure of the extracellular matrix receptor is retained. For example, it is desirable to use an integrin-immunoglobulin chimeric protein heterodimer complex obtained by modifying the heterodimer structure of an integrin to allow its α and β chains to be covalently associated with each other. As the integrin-immunoglobulin chimeric protein heterodimer complex, it is preferable that the α chain of the integrin is $\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 9, \alpha v, \alpha L, \alpha M, \alpha X, \alpha 11b$ or αE , and among them, $\alpha 2$ is more preferable. Furthermore, it is preferable that the β chain is $\beta 1, \beta 2, \beta 3, \beta 4, \beta 5, \beta 6, \beta 7$ or $\beta 8$, and among them, $\beta 1$ is more preferable. It is further more preferable that the α chain is $\alpha 2$ and that the β chain is $\beta 1$. The platelet substitute of the present invention is described below mainly in reference to a typical extracellular matrix receptor, integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex, but the present invention is not limited thereto or thereby.

To confirm the applicability of a purified integrin-

immunoglobulin chimeric protein heterodimer complex as a drug, the purified protein itself is used for examining its pharmacological activity. For obtaining higher capability of being bound to an extracellular matrix, it is more preferable to use an integrin-immunoglobulin chimeric protein heterodimer complex bound to a carrier such as a lipid or protein polymer, etc., but the present invention is not limited to this method.

See D24 For use as a platelet substitute, it is preferable to bind an integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex to a liposome covalently according to the method stated in a report (Martin, F. J. et al., Biochemistry, 20, 4229 (1981)). The carrier can also be any other drug carrier than a liposome as far as its use for drugs is permitted. If a liposome is used as the carrier, the liposome is prepared according to the composition and method stated in a published book "Preparation and Experiments of Liposomes (in Japanese)", Oku, N. (1994), Hirokawa Shoten), but a preferable method is such that the epitope bound to the extracellular matrix of an integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex is exposed outside the liposome membrane.

See D25 For confirming that an integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex is bound on the prepared liposome carrier, a flow cytometer is used. The reagents which can be used for recognizing the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex include

an anti-integrin $\alpha 2$ antibody, anti-integrin $\beta 1$ antibody, anti-human immunoglobulin antibody, etc. If the antibody used is fluorescently labeled, it can be used for determination directly, but if it is not fluorescently labeled, a secondary antibody which recognizes the immunoglobulin class of the animal species used for preparing the antibody is used as a fluorescent label. As a further other confirmation method, the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer composite itself can be labeled by an enzyme or radioisotope, etc., for confirmation in proper combination with a color dye or radioactivity measuring instrument, etc.

Sub D25 cont

Sub D26 To examine the extracellular matrix binding capability using an integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome, it is preferable to suspend the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome into a buffer with a physiological cation concentration or plasma. The buffer with a physiological cation concentration refers to a buffer containing at least cations such as Mg ions or Ca ions and adjusted to about neutrality. The plasma is prepared by processing the blood collected in the presence of an anticoagulant, according to a general plasma preparation method. As the anticoagulant, for example, heparin or EDTA solution can be added by sufficient units. A marketed normal plasma, coagulation factor deficient plasma or serum, etc. can also be used. However, if the

anticoagulant used lowers the cation concentration, cations are added to achieve a physiological concentration later. Then, the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome is mixed with an extracellular matrix or its fragment coated on a carrier for a certain time, to judge whether binding takes place. It is preferable that the coating of the extracellular matrix or its fragment as a solid phase is achieved by using a plastic plate, etc., but marketed beads for coating an extracellular matrix as a solid phase, etc. can also be used. When a collagen is used as the extracellular matrix, any animal species and type can be used. The binding reaction between an integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome and an extracellular matrix is effected according to a general method adopted for observing the adherence reaction of platelets. In many cases, they are allowed to stand mainly in a static system for a certain time, to induce binding to the matrix, but it is preferable to apply a shaking or shear stress, etc.

See D27 The integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome is bound to an extracellular matrix under the conditions as described above, and the amount of binding is measured by applying the above mentioned ELISA method using an anti-human immunoglobulin antibody. For more accurate determination, it is desirable to immobilize the liposome bound to the matrix by 1% glutaraldehyde, etc. As

another method than the ELISA method, for example, if a radio-labeled lipid is incorporated into the liposome beforehand, the amount of the liposome bound to the extracellular matrix can be obtained as radioactivity. Furthermore, to qualitatively judge the binding and covering degree to the extracellular matrix, a labeled antibody for recognizing the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex on the bound liposome can be combined with a color dye, etc., to dye the portions where the liposome is bound. It is more preferable that the generally used tissue antibody dyeing method is used to use a peroxidase labeled antibody against the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex and diaminobenzidine in combination, but the measuring method is not limited to it. As a further other method, the area covering the extracellular matrix can be obtained as a covering rate using an image processing analyzer.

Sub D28 Methods for examining the hemostasis of platelets include testing the adhering capability of platelets to the extracellular matrix and the agglutination capability induced by a collagen ("Handbook on the Examination of Blood Coagulation (in Japanese)", p. 65-78, Fukutake, M. and Fujimaki, M. (1987), Uchido Yagi Shoten, Santro, S.A., Cell, 46, 913-920 (1986), Lethagen, S. and Rugarrn, P., Thrombo Haemost., 67, 185-186 (1982)). Especially the adhering

capability of platelets to the extracellular matrix is an indicator of primary hemostasis. The adhering capability is evaluated by using blood as it is, or platelet rich plasma or platelets washed by a buffer with physiological ions. Therefore, whether or not the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome obtained in the present invention can be a functional substitute of platelets can be judged in reference its binding capability and the level of the binding capability to the extracellular matrix in the existence of plasma components or at a physiological ion concentration.

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Sue D28 cont
Sue D29
If the binding capability of the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome obtained in the present invention to the extracellular matrix in the presence of the plasma components is strong, it suggests that the liposome can be a platelet substitute. Therefore, it can be used as a therapeutic or preventive agent against the congenital and acquired bleeding tendency due to platelet abnormality, and also widely as a platelet transfusion substitute.

Sue D30
Similarly the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome obtained in the present invention can be a therapeutic or preventive agent for conditions of diseases where vascular endothelial cell disorder is a problem. For example, it was reported that in

the prognosis of PTCA (percutaneous coronary restenosis), the excessive accumulation of platelets on the extracellular matrix exposed by balloon catheter treatment triggers restenosis (Liu, M.W. et al., Circulation, 79, 1374-1378 (1989)). In Example 22, the effect of the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome to cover the extracellular matrix was confirmed, and this effect can reduce the excessive accumulation of platelets to allow use also as a restenosis preventive. Furthermore, if the integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome is labeled by a medically allowable method, it can be used for monitoring the region of the extracellular matrix exposed by vascular endothelial cell injury, and furthermore, if a drug is enclosed in the liposome, it can also be applied to the targeting therapy for a local injured region.

See 31
When any integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex liposome stated in the present invention is used as a platelet substitute, the administration paths include infusion, intravenous administration, etc., and it is usually used by being suspended in any physiologically suitable solution such as a salt solution or plasma, etc. It can be used alone or also in combination with another chimeric protein heterodimer complex with an extracellular matrix receptor or its immunoglobulin. It can also be used together

with another drug containing total platelets. The dose is properly selected to suit the symptom, age, body weight, etc., and can be 0.1 mg to 10 g per day as the amount of the protein for an adult, being able to be administered at a time or in several times. It can also be mixed with a pharmaceutically allowed carrier or excipient, etc., to be applied locally to the injured region as an externally applied drug such as an ointment, liniment or plaster. In this case, the externally applied drug is prepared to be 1 ng/cm² to 1 mg/cm² as the amount of the protein per one time of coating.

Examples

To describe the present invention in more detail, examples are given below. The general methods of recombinant DNA experiments conformed to those stated in a published book ("Antibody", Harlow, E. and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York).

Example 1

Construction of human IgG₁ heavy chain expression vector

See D32 As human IgG₁ genome gene, a clone identical with reported base sequence information (Ellison, J. W. et al., Nucleic Acids Res., 10, 4071-4079 (1982)) was acquired from a human genomic library (CLONTECH) using a hybridization cDNA probe based on the sequence information. This was used as the template DNA for PCR. As primers for amplifying the DNA fragment containing the hinge region (H) and the constant

region portions (CH2 and CH3) of human IgG₁ gene, a DNA oligomer shown in sequence No. 4 of the sequence table (hereinafter a sequence No. of the sequence table is simply called a sequence No.) with BamH I restriction site and a DNA oligomer shown in sequence No. 5 with Xba I restriction site were synthesized.

5'-GCGGATCCCGAGCTGCTGGAAGCAGGCTCAG-3' (Sequence No. 4)

5'-CCTCTAGACGGCCGTCGCACTCATTTA-3' (Sequence No. 5)

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The template DNA, primers, dNTPs (an equimolar mixture of dATP, dCTP, dGTP and dTTT) and Taq polymerase (Takara) were mixed in a PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin, pH 8.3), and in a thermal cycler (Perkin Elmer Cetus), the mixture was treated at 94 °C for 1 minute for DNA denaturation, at 58°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA was digested by restriction enzymes BamH I and Xba I, and the DNA fragment was purified by 1% agarose gel according to a general method ("Antibody", Harlow, E. and Lane, D. (1988), Cold Spring Harbor Lab. Press, New York). It was linked, using a T4DNA ligase, with a large DNA fragment of pBluescriptSK(+) (STRATAGENE) purified and digested by restriction enzymes BamH I and Xba I. The plasmid DNA was used to transform Escherichia coli (JM109), and the transformant was selected, to obtain a plasmid DNA (IgG₁ Bluescript). Then, expression

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vector pcDL-SR α 296 was digested by restriction enzyme BamH I, and blunted at the termini by T4DNA polymerase treatment, and a Not I linker was linked. The large DNA fragment obtained by digesting it by restriction enzymes Not I and Xho I and the small DNA fragment obtained by digesting IgG₁ Bluescript by restriction enzymes Not I and Xho I were purified according to a general method, and linked by T4DNA ligase. It was transformed into Escherichia coli (HB101), and the transformant was selected, to obtain a plasmid DNA. Hereinafter this plasmid (IgG₁SR α) is called human IgG₁ expression vector. In the following examples, since the basic protocol of gene manipulation is the same as above, the description will be simplified.

Example 2

Construction of integrin α 4-IgG heavy chain chimeric protein expression vector

July 33 The DNA fragment coding for the extracellular portion of integrin α 4 was obtained by cloning based on reported cDNA sequence information (Takada, Y. et al., EMBO J., 8, 1361-1368 (1989)). The restriction site EcoR I of 1801-base-position of sequence No. 1, the restriction site Stu I of 112-base-position and the restriction site BamH I of 2949-base-position were used for linking the region from the N terminus translation initiation site to Stu I cut site as α 4-1, the region from Stu I cut site to EcoR I cut site as α 4-2, and the

region from EcoR I detached site to BamH I detached site as α 4-3. The detailed methods are described below.

July 34 The portion coding for α 4-1 was designed to be cloned by linking the DNA oligomers of sequence Nos. 6 to 9, and the DNA oligomers shown in sequence Nos. 6 to 9 were synthesized. For the sequence Nos. 6 and 7, restriction site Xba I was added on the side to code for the N terminus, for linking to a vector. Furthermore, compared with the known sequence information, the bases at the 60-, 63- and 64-positions were substituted from C to T, C to A and C to G respectively, and the bases at the 112- and 114-positions were substituted from C to A and C to G respectively. Because of substitution at the 112- and 114-position, restriction site Stu I was inserted on the side to code for the N terminus of sequence Nos. 8 and 9. The 5' termini of the synthesized oligomers were phosphorylated and annealed, and were linked using T4DNA ligase. After completion of linking, restriction enzymes Xba I and Stu I were used for cutting, and electrophoresis was effected by 5% agarose (NuSieve GTGagarose, FMC) gel. The intended DNA fragment (α 4-1) of about 120 bp was cut out and purified.

5' - CTAGACCACCATGTTCCCCACCGAGAGCGCATGGCTTGGGAAGCGAGGCGCGAACC CGGCCCGGA
GCTGCA-3' (Sequence No. 6)

5' - GCTTCGGGGCCCGGGTTCGCGCCTCGCTTCCCAAGCCATGCGCTCTCGGTGGGGAACATGGTGGT-3'
(Sequence No. 7)

5' - CTCGGGGAGACGGTGATGCTGTTGCTGTGCCTGGGGTCCCCACCGGCAGG-3'

(Sequence No. 8)

5' - CCTGCCGGTCGGGACCCCCAGGCACAGCAACAGCATCACCGTCTCCCGGAGTCGA-3'

(Sequence No. 9)

Then, the RNA of human osteosarcoma cell line MG63 (ATCC CRL 1427) as an integrin $\alpha 4$ expressing cell was separated, and PolyA(+) RNA was purified using oligo dT cellulose column (NEB). Based on it, a single stranded cDNA was synthesized using a reverse transcriptase (GIBCO), and used as the template for PCR. As primers for amplifying $\alpha 4$ -2 and $\alpha 4$ -3 DNAs, four DNA oligomers of sequence Nos. 10 to 13 with Pst I and Stu I restriction sites inserted (sequence No. 10) or BamH I restriction site inserted (sequence No. 13) were synthesized.

5' - CACTGCAGGCAGGCCTTACAACGTGGACACTGAGAGC-3' (Sequence No. 10)

5' - GCAGAAACCTGTAAATCAGCAG-3' (Sequence No. 11)

5' - GCATTTATGCGGAAAGATGTGC-3' (Sequence No. 12)

5' - CGGGATCCGTGAAATAACGTTTGGGTCT-3' (Sequence No. 13)

The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer, and the mixture was treated by a thermal cycler at 94°C for 1 minute for DNA denaturation, at 58°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA fragments of $\alpha 4$ -2 and $\alpha 4$ -3 were digested by Pst I and EcoR I respectively, or EcoR I

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and BamH I and sub-cloned into pBluescriptKS(+) (STRATAGENE), to prepare plasmid DNAs (hereinafter called $\alpha 4-2$ Bluescript and $\alpha 4-3$ Bluescript). Then, upstream of the $\alpha 4-2$ Bluescript, $\alpha 4-1$ was linked using Xba I and Stu I restriction sites, to prepare a plasmid DNA (hereinafter called $\alpha 4-1-2$ Bluescript).

Sub D36
The $\alpha 4-1-2$ Bluescript was digested by restriction enzyme Not I, and blunted at the termini by T4DNA polymerase treatment, being digested by restriction enzyme EcoR I, to prepare a small DNA fragment. The $\alpha 4-3$ Bluescript was digested by restriction enzymes EcoR I and BamH I, to prepare a small DNA fragment. The two small DNA fragments were simultaneously linked to a large DNA fragment obtained by digesting IgG₁SR α by restriction enzymes EcoR V and BamH I, to obtain a plasmid DNA. The obtained base sequence coding for integrin $\alpha 4$ ·IgG heavy chain chimeric protein is shown as sequence No. 1. The plasmid (integrin $\alpha 4$ ·IgGSR α) is hereinafter called integrin $\alpha 4$ ·IgG heavy chain chimeric protein expression vector.

Example 3

Construction of $\beta 1$ ·IgG heavy chain chimeric protein expression vector

Sub D37
The RNA of human fibroblast cell line MRC5 (ATCC CCL 171) as an integrin $\beta 1$ expressing cell was separated, and oligo dT cellulose column was used to purify PolyA (+)RNA. Based on it, a single stranded cDNA was synthesized using a reverse

transcriptase, and used as the template for PCR. As primers, two DNA oligomers of sequence Nos. 14 and 15 with BamH I site (sequence No. 15) inserted on the side coding for C terminus were synthesized according to the sequence information (Cott Argraves, W. et al., J. Cell Biol., 105, 1183-1190 (1987)).

5' - GCGGAAAAGATGAATTTACAAC-3' (Sequence No. 14)

5' - GTGGGATCCTCTGGACCAGTGGGACAC-3' (Sequence No. 15)

Sub D38 The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer, and treated by a thermal cycler at 94°C for 1 minute for DNA denaturation, at 57°C for 2 minutes for annealing the primers and at 72°C for 3 minutes for elongating the primers. This treatment was performed 30 cycles. The amplified DNA was blunted at the termini by T4DNA polymerase treatment, and digested by restriction enzyme BamH I. Then, the DNA fragment was purified. Subsequently, the DNA fragment obtained in PCR before was sub-cloned at the Sma I and BamH I sites of pBluescriptKS(+). A small DNA fragment purified by digesting it by restriction enzymes EcoR I and BamH I was inserted into a large DNA fragment of IgG₁SR α treated by restriction enzymes EcoR I and BamH I, to obtain a plasmid DNA. The obtained base sequence coding for $\beta 1$ -IgG heavy chain chimeric protein is shown in sequence No. 2. The plasmid (integrin $\beta 1$ -IgGSR α) is hereinafter called integrin $\beta 1$ -IgG heavy chain chimeric protein expression vector.

Example 4

Transfection of $\alpha 4$ - IgG heavy chain chimeric protein expression vector and $\beta 1$ - IgG heavy chain chimeric protein expression vector into animal cells, and their expression

Integrin $\beta 1$ - IgGSR α as $\beta 1$ - IgG heavy chain chimeric protein expression vector and pSV2dhfr (BRL) were mixed at a ratio of 10 : 1, and the mixture and lipofectin reagent (GIBCO BRL) were gently mixed and allowed to stand at room temperature for 15 minutes. The mixture was added dropwise to dihydrofolic acid reductase deficient CHO cells (ATCC CRL 9096). After 18 hours of dropwise addition, the mixture was cultured in a medium (10%FBS (GIBCO), nucleic acid-containing α MEM medium (GIBCO BRL)) for about 2 days, and the cells were dispersed by trypsin-EDTA treatment. The cells were suspended in a first selective medium (10% FBS-containing nucleic acid-free α MEM medium (GIBCO BRL)), and the suspension was disseminated into a 96-well plate (CORNING), for selective culture for about 10 days. Then, the amount of integrin $\beta 1$ - IgG heavy chain chimeric protein produced in the culture supernatant was determined according to the ELISA method (described later), and the clone showing the highest production was stabilized by cloning according to the limiting dilution method.

Then, into the stabilized integrin $\beta 1$ - IgG heavy chain chimeric protein producing CHO cells, the integrin $\alpha 4$ - IgG heavy chain chimeric protein expression vector was transfected

Example 5

Fifty microliter per well of anti-human integrin $\alpha 4$

antibody (Becton & Dickinson, Clone L25.3) or anti-human integrin $\beta 1$ antibody (Coulter, Clone 4B4) ($12 \mu\text{g/ml}$ each) was put into a 96-well immunoplate (NUNC), and allowed to stand at 4°C for 16 hours. Then, each well was washed by Dulbecco's phosphate buffered saline (Nissui Seiyaku, not containing Ca or Mg ions, hereinafter called PBS(-)) twice, and non-specific reaction was blocked by PBS(-) containing 25% Block Ace (Snow Brand Milk Products Co., Ltd.). After blocking, the culture supernatant of CHO cells grown in selective medium was properly diluted, and reacted with the coated antibody at room temperature for 1 hour. After the reaction, the surface of the plate was washed with 0.02% Tween-containing PBS(-) (hereinafter called T-PBS) twice. It was then caused to react with biotinated anti-human IgG antibody (Vector) for 1 hour, and the reaction mixture was washed with T-PBS twice, and in succession caused to react with avidin-horseradish peroxidase (Sigma) for 1 hour. The reaction mixture was washed with PBS(-) twice. The PBS(-) was perfectly aspirated, and orthophenylenediamine was used as a substrate for color development. The absorbance at 490 nm were measured using a microplate reader (Bio-rad NOVAPATH), and the clone showing a high absorbance value was selected.

Example 6

Purification of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex

(1) Culture of CHO cells and preparation of cultured supernatant solution

Sub 40 The CHO cells highly capable of producing the $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex were cultured in nucleic acid-free αMEM medium containing 5% FBS (Ultra-low IgG grade, GIBCO) (hereinafter called $\alpha \text{MEM}(-)$ medium, GIBCO BRL) for one day, to reach semiconfluent, and they were cultured in $\alpha \text{MEM}(-)$ medium containing 1% FBS (Ultra-low IgG grade) for 3 days, and the culture supernatant was collected. It was concentrated to 1/10 volume by ultrafiltration using Prep-scale (Millipore), and 1M Hepes solution (pH 8.0) was added to achieve a final concentration of 5 mM, for preparing a starting solution for further purification.

(2) Protein A column chromatography

The starting solution for further purification was passed through Prosep Guard column (bioPROCESSING), and applied to Prosep A column (bioPROCESSING). After completion of application, it was washed with 10 times the column volume of PBS(-), and the proteins were eluted at a pH 6 - 3 gradient of 0.1M citrate buffer solutions. The peak fraction eluted at pH 3 was collected, and 1M Tris-HCl solution (pH 8.5) was added by 0.1 volume for neutralization. The solution was dialyzed against PBS(-).

(3) Affinity column chromatography

Sub D41
FMP activated Cellulofine (Seikagaku Kogyo) was equilibrated by a coupling buffer (50 mM Na₂CO₃-NaHCO₃ pH 8.5), and a peptide showing sequence No. 3 (hereinafter called CS-1 peptide) synthesized by a peptide synthesizer was added. The mixture was inverted and mixed at 4 °C for 16 hours.

Cys Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr (Sequence No. 3)
After completion of mixing, the mixture was washed with the coupling buffer, and a blocking buffer (0.1 mM monoethanolamine, 50 mM Tris-HCl, pH 8.0) was added. The mixture was inverted and mixed further at room temperature for 6 hours. Then, the mixture was sufficiently washed with TBS solution (150 mM NaCl, 20 mM Tris-HCl, 1 mM MnCl₂, pH 7.5), to prepare CS-1 peptide bound Cellulofine column. To the column the starting solution for further purification was applied and allowed to stand at room temperature for 3 hours, and washed with 10 times the column volume of a washing buffer (1M NaCl, 0.1% Triton, 20 mM Tris-HCl, 1 mM MnCl₂, pH 7.5) and the same volume of the TBS solution. After completion of washing, an elution buffer (10 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5) was used, to elute the proteins bound to the CS-1 column. The eluate was collected and dialyzed against PBS(-).

(4) SDS-PAGE

Sub D42
The eluted fractions of (3) were subjected to SDS-PAGE under non-reducing or reducing condition using 6.0 or 7.0% acrylamide gel, and the gel was stained with Coomassie-blue.

As a result, under non-reducing condition, two bands considered to be attributable to the $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex and its polymer were observed. Under reducing condition, two bands (170 kDa and 135 kDa) considered to be attributable to the integrin $\alpha 4 \cdot \text{IgG}$ heavy chain chimeric protein and the integrin $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein and two bands (80 kDa and 90 kDa) considered to be attributable to the intramolecular cleavage of the integrin $\alpha 4 \cdot \text{IgG}$ heavy chain chimeric protein (Hemler, M. E. et al., J. Biol. Chem., 262, 11478-11485 (1987)) were observed. These results suggest that the eluted protein of (3) has a molecular structure considered to be $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and that the molecules constituting the heterodimer are linked by a disulfide bond between the IgG heavy chains.

Example 7

Identification of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and examination of its structural stability

(1) Immunoprecipitation using anti-integrin antibodies and influence of a cationic chelating agent

Sub-D13 The basic method conformed to a published book ("Antibodies", Harlow, E. et al., (1988), Cold Spring Harbor Lab. Press, New York). That is, the eluted protein of Example

6 (3) considered to be $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex was ^{125}I -labeled using the lactoperoxidase method. Then, Affigel-10 (Bio-rad) was washed with 0.1 M Hepes solution (pH 8.0), and normal murine IgG, anti-human integrin $\alpha 4$ antibody (clone 11C2B) and anti-human integrin $\beta 1$ antibody (clone 4B4) were added. Reaction was effected at 4°C for 16 hours to cause covalent bonding, to prepare normal murine IgG beads and the respective antibody beads. Then, the ^{125}I labeled $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex and normal murine IgG beads were inverted and mixed at 4 °C for 4 hours for preclearing, and the mixture and the antibody beads were inverted and mixed at 4°C for 16 hours. After completion of mixing, the beads were washed with a washing buffer (200 mM Tris-HCl, 0.5 M NaCl, 0.1% NP-40, 1 mM MgCl_2 or 10 mM EDTA, pH 8.0) three times. After completion of washing, a sample buffer for electrophoresis was added to the beads for treatment at 100 °C for 5 minutes, and the mixture was centrifuged. The supernatant solution was analysed by electrophoresis under reducing condition. After completion of electrophoresis, the gel was dried by a gel dryer, and the protein was detected by autoradiography.

As a result of immunoprecipitation in the presence of 1 mM MgCl_2 , from the beads of both the anti-human integrin $\alpha 4$ antibody and the anti-human integrin $\beta 1$ antibody, the same

precipitation patterns expected from the structure of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex were obtained. Thus, the protein obtained in (3) of Example 6 was identified as $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex.

Sub 714
Sub 719
On the other hand, the immunoprecipitation pattern obtained by using the anti-integrin $\beta 1$ antibody beads in the presence of 10 mM EDTA was the same as that in the presence of 1 mM MgCl_2 , to clarify that the association between integrin $\alpha 4 \cdot \text{IgG}$ heavy chain chimeric protein and integrin $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein does not depend on cations. The above results suggest that the eluted protein obtained in (3) of Example 6 was certain $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and if the result of (4) of Example 6 is also taken into account, it is strongly suggested that the association between both the proteins is stable association through a disulfide bond existing the IgG heavy chains.

Sub 710
(2) Examination on the structural stability of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex by sequential immunoprecipitation

According to (1), ^{125}I labeled $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex was caused to react with normal murine IgG beads, anti-human integrin $\alpha 4$ antibody (11C2B) beads or anti-human integrin $\beta 1$ antibody

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B64TTF "H FGGG60

(4B4) beads at 4°C for 4 hours, and the reaction mixture was washed. After washing, the reaction mixture was boiled at 100°C for 5 minutes in the presence of 2% SDS and centrifuged, and the supernatant (primary immunoprecipitation sample) was diluted to 10 times by 1% BSA-containing PBS, and was again reacted with the anti-integrin $\beta 1$ antibody beads and the anti-integrin $\alpha 4$ antibody beads at 4°C for 16 hours. After completion of reaction, the beads were washed, and a sample buffer for electrophoresis was added. The mixture was treated at 100°C for 5 minutes and centrifuged. The supernatant solution (secondary immunoprecipitation sample) was analyzed by SDS-PAGE/autoradiography.

As a result, the electrophoretic pattern obtained by the primary immunoprecipitation was also similarly observed in the secondary immunoprecipitation. This result suggests that the association between the $\alpha 4 \cdot \text{IgG}$ heavy chain chimeric protein and the $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein in the $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex is not dissociated either by boiling in the presence of 2% SDS, and strongly supports that the complex has a stable heterodimer structure based on a disulfide bond.

Example 8

Sub D45
Binding of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex to VCAM-1

It was examined that the $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$

heavy chain chimeric protein heterodimer complex produced by CHO cells can be bound to the ligand of integrin $\alpha 4 \beta 1$ by using the cells expressing VCAM-1. Human normal umbilical intravenous endothelial cells were cultured with IL-1 3U/ml for 16 hours, to prepare VCAM-1 expressing cells. The cells were treated by 1 mM EDTA at 37°C for 15 minutes, for dispersion as single cells. The cells (2×10^5 cells per sample tube) were cultured with the supernatant of the CHO cells producing $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex for 30 minutes in the presence of 1 mM (final concentration) MnCl_2 or 3 mM (final concentration) EDTA. After completion of reaction, the cells were washed twice by centrifugation at 1200 rpm at room temperature for 5 minutes using a buffer for binding assay (24 mM Tris-Cl, 10 mM Hepes, 150 mM NaCl, 1 mM MnCl_2 or 1 mM EDTA, 1% BSA, 2 mM glucose, pH 7.4). After washing, FITC labeled-anti-human IgG antibody (Cappel) was added, and incubated at room temperature for 20 minutes. The cells were washed by the same buffer, and the chimeric proteins bound to the cells were determined by a flow cytometer (ELITE, Coulter).

The results are shown in Fig. 1. It was observed that the fluorescence intensity showing the binding of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex increased by culturing the VCAM-1 expression cells with the supernatant containing $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain

chimeric protein heterodimer complex. The binding was inhibited by adding anti-human integrin antibodies (anti- α -antibody: clone L25.3, 10 μ m/ml + anti- β 1 antibody: clone 4B4, 10 μ m/ml) or 3 mM EDTA. This result suggests that the α 4 \cdot IgG heavy chain- β 1 \cdot IgG heavy chain chimeric protein heterodimer complex can be bound to VCAM-1 like the integrin α 4 β 1 existing on the surfaces of cell membranes, and furthermore that the binding is α 4 β 1-specific and retains a feature of the binding that it is dependent on cations.

Example 9

Binding of α 4 \cdot IgG heavy chain- β 1 \cdot IgG heavy chain chimeric protein heterodimer complex to a peptide fragment of fibronectin

Sub 46 The capability of α 4 \cdot IgG heavy chain- β 1 \cdot IgG heavy chain chimeric protein heterodimer complex to be bound to the peptide fragment (sequence No. 3) of the other ligand, fibronectin was also examined.

Sub 47 At first, according to the said report (Humphries, M. J. et al., J. Biol. Chem., 262, 6886-6892 (1987)), the peptide fragment of sequence No. 3 (CS-1 peptide) was bound to rabbit IgG (Sigma), to prepare CS-1-IgG. The CS-1-IgG was diluted by PBS(-), and put in a 96-well immunoplate (NUNC) by 100 μ l/well, and allowed to stand at 4°C for 16 hours, to be formed as a solid phase on the plate.

Sub 48 After completion of standing, the surface of the plate was

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washed with PBS(-) twice and treated with denatured 1% BSA(heat-natured at 80°C for 10 minutes)-PBS solution (300 μ l/well) at 4°C for 3 hours to block the nonspecific reaction. Then, the solid phase CS-1-IgG and the CHO culture supernatant (100 μ l) containing α 4-IgG heavy chain- β 1-IgG heavy chain chimeric protein heterodimer complex were reacted with each other at 30°C for 3 hours. The non-bound α 4-IgG heavy chain- β 1-IgG heavy chain chimeric protein heterodimer complex was removed by washing with 0.1% BSA-containing TBS buffer (150 mM NaCl, 25 mM Tris-HCl, 1 mM $MnCl_2$, pH 7.4) twice, and the bound α 4-IgG heavy chain- β 1-IgG heavy chain chimeric protein heterodimer complex was detected by biotin labeled anti-human IgG antibody (Vector) as the primary antibody and avidin labeled horseradish peroxidase (Sigma) as the secondary antibody. The surface of the plate was washed with the TBS buffer. Orthophenylenediamine was added as a substrate to it for color development, and the absorbance at 490 nm were measured.

The results are shown in Fig. 2. The reaction with α 4-IgG heavy chain- β 1-IgG heavy chain chimeric protein heterodimer complex showed a rise in the absorbance indicating the binding to CS-1 peptide. The binding was almost perfectly inhibited by the presence of anti-integrin α A antibody (clone L25.3), anti-integrin β 1 antibody (clone 4B4) or 5 mM EDTA. Therefore, it was clarified that α 4-IgG heavy chain- β 1-IgG

heavy chain chimeric protein heterodimer complex can be bound also to the CS-1 peptide which is a peptide fragment of fibronectin, and that a feature of binding that it depends on cations is retained.

Example 10

Evaluation of an inhibitory peptide by using a system for determining the binding of $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex to a peptide fragment of fibronectin

Sub 149 In the binding determination system of Example 9, the effects of three peptides, i.e., sequence No. 16 (hereinafter called GPEILDVPST), 17 (hereinafter called GPEILEVPST) and 18 (hereinafter called GRGDSP) were examined.

Gly Pro Glu Ile Leu Asp Val Pro Ser Thr (Sequence No. 16)

Gly Pro Glu Ile Leu Glu Val Pro Ser Thr (Sequence No. 17)

Gly Arg Gly Asp Ser Pro (Sequence No. 18)

Sub D50 The all peptides were synthesized by a peptide synthesizer. The peptide and 100 μl of CHO cultured supernatant solution containing $\alpha\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex were mixed at room temperature for 20 minutes, and the binding to CS-1-IgG was determined according to the method in Example 9. The results are shown in Fig. 3. GPEILDVPST showed temperature-dependent inhibitory activity in a range of 0.1 to 10 $\mu\text{mg/ml}$, but GPEILEVPST and GRGDSP did not show any inhibition of the

binding. These results show that the binding determination system in Example 9 allows to detect the inhibiting effect of the peptide (GPEILDVPST) inhibiting the binding between integrin $\alpha 4 \beta 1$ and CS-1 peptide specifically .

Example 11

Construction of integrin $\alpha 2$ - IgG heavy chain chimeric protein expression vector

Sub D51 The DNA fragment coding for the extracellular portion of integrin $\alpha 2$ was divided into $\alpha 2$ -1 and $\alpha 2$ -2 based on the reported cDNA sequence information (Takada, Y. et al., J. Cell. Biol., 109, 397-407 (1989)) and subcloned, and they were integrated on an expression vector. At first, the RNA of human fibroblast cell line MRC-5 (ATCC CCL 171) as integrin $\alpha 2$ expressing cell was separated, and an oligo dT cellulose column was used to purify PolyA(+)RNA. Based on it, a single stranded cDNA was synthesized and used as the template of PCR. As PCR primers, DNA oligomers of sequence Nos. 20 and 21 were synthesized for $\alpha 2$ -1, and DNA oligomers of sequence Nos. 22 and 23, for $\alpha 2$ -2.

5' -GCTCGAGCAAACCCAGCGCAACTACGG-3' (Sequence No. 20)

5' -ATAGTGCCCTGATGACCATTG-3' (Sequence No. 21)

5' -GATGGCTTTAATGATGTGATTG-3' (Sequence No. 22)

5' -TGTTGGTACTTCGGCTTTCTC-3' (Sequence No. 23)

Sub D52 The template cDNA, primers, dNTPs and Taq polymerase were mixed in a PCR buffer and PCR was performed 30 cycles by a

thermal cycler (reaction conditions: 94°C 1 minute - 60°C 2 minutes - 72°C 3 minutes). The amplified DNA fragment of $\alpha 2-1$ was digested by restriction enzymes Xho I and EcoR I, and the DNA fragment of $\alpha 2-2$ was blunted at the termini by T4DNA polymerase treatment and digested by restriction enzyme EcoR I. Each fragment was purified. The two purified DNA fragments were caused to react in a phosphating reaction solution (50 mM Tris-HCl, 10 mM MgCl₂, 25 mM DTT, 1 mM ATP, 0.1 U/ μ l T4 polynucleotide kinase (Takara), pH 8.0) at 37 °C for 1 hour, and the reaction mixture was heat-treated at 68°C for 5 minutes to inactivate the enzyme. Then, IgG₁SR α prepared in Example 1 was digested by restriction enzyme BamH I and caused to react in Klenow reaction solution (66 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 0.2 mM dNTPs, 0.05 U/ μ l Klenow fragment (Takara), pH 7.5) at 37°C for 30 minutes, to blunt the termini, and the reaction mixture was heat-treated at 70°C for 5 minutes to inactivate the enzyme. Furthermore, a large DNA fragment was digested by restriction enzyme Xho I, and purified. The two ($\alpha 2-1$ and $\alpha 2-2$) DNA fragments phosphated before were inserted into the large DNA fragment, to obtain a plasmid DNA. The obtained base sequence coding for integrin $\alpha 2$ · IgG heavy chain chimeric protein is shown in sequence No. 19. This plasmid (integrin $\alpha 2$ · IgGSR α) is hereinafter called $\alpha 2$ · IgG heavy chain chimeric protein expression vector.

Example 12

Transfection of integrin $\alpha 2$ · IgG heavy chain chimeric protein expression vector and integrin $\beta 1$ · IgG heavy chain chimeric protein expression vector into animal cells, and their manifestation

The integrin $\alpha 2$ · IgG heavy chain chimeric protein expression vector was transfected into the integrin $\beta 1$ · IgG heavy chain chimeric protein producing CHO cells prepared and stabilized in Example 4, according to the lipofectin method described in Example 4. That is, integrin $\alpha 2$ · IgGSR α and pSV2neo (BRL) were mixed at 10 : 1, and the mixture was mixed with lipofectin reagent. The mixture was added dropwise to the cells. Eighteen hours after completion of dropwise addition , the mixture was cultured in a first selective medium for 2 days, and the cells were dispersed by trypsin-EDTA treatment. The cells were suspended in a second selective medium, and the suspension was disseminated into a 96-well plate. Resistant cells were selectively cultured for about 10 days. Then, the amount of integrin $\alpha 2$ · IgG heavy chain chimeric protein and the amount of integrin $\beta 1$ · IgG heavy chain chimeric protein produced in the culture supernatant were determined according to the ELISA method (described later), and a clone producing almost the same amounts of both the chimeric proteins was picked up. The clone was cloned twice according to the limiting dilution analysis, to be stabilized as a clone capable of producing α

2 · IgG heavy chain- β 1 · IgG heavy chain chimeric protein heterodimer complex.

Example 13

Determination of the amounts of integrin α 2 · IgG heavy chain chimeric protein and integrin β 1 · IgG heavy chain chimeric protein by the ELISA method

53 Fifty microliter per well of anti-human integrin α 2 antibody (Becton & Dickinson, clone P1E6) or anti-human integrin β 1 antibody (clone 4B4) ($2\mu\text{g/ml}$ each) was put into a 96-well immunoplate, and allowed to stand at 4°C for 16 hours. Then, each well was washed with PBS(-) twice, blocked, and the culture supernatant of the CHO cells grown in second selective medium was properly diluted and reacted with the coated-antibody at room temperature for 1 hour. After the reaction, the surface of the plate was washed with T-PBS twice, and caused to react with biotinated anti-human IgG antibody for 1 hour and with avidin-horseradish peroxidase for 1 hour, and the reaction mixture was washed with PBS(-) twice. After completion of reaction, orthophenylenediamine was used as a substrate for color development, and the absorbance values at 490 nm were measured using a microplate reader. A clone showing a high absorbance value was selected.

Example 14

Purification of α 2 · IgG heavy chain- β 1 · IgG heavy chain chimeric protein heterodimer complex

(1) Culture of CHO cells and preparation of cultured supernatant solution

Sub D54 The CHO cells highly capable of producing $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex were cultured in an $\alpha \text{MEM}(-)$ medium containing 5% FBS (Ultra-low IgG grade) for 1 day, to reach semiconfluent, and they were cultured on an $\alpha \text{MEM}(-)$ medium containing 1% FBS (Ultra-low IgG grade) for 3 days. The culture supernatant was collected, and concentrated to 1/10 volume by ultrafiltration. Then, 1M Hepes solution (pH 8.0) was added to achieve a final concentration of 5 mM, to obtain a starting solution for further purification.

(2) Protein A column chromatography

The starting solution for further purification was passed through Prosep Guard column, and applied to Prosep A column. After completion of application, it was washed with 10 times the column volume of PBS (-), and in succession, the proteins were eluted at a pH 6 to 3 gradient of 0.1M citrate buffers. The peak fraction eluted at pH 3 was collected, and 1M Tris-HCl solution (pH 8.5) was added by 0.1 volume for neutralization. The mixture was dialyzed against PBS(-).

(3) Affinity column chromatography

According to a report (Kirchhofer, D. et al., J. Biol. Chem., 265, 615-618 (1990)), a collagen immobilized column with a collagen (Type I, Sigma) coupled to cyanogen-bromide-

activated Sepharose (Sigma) was prepared. Then, the starting solution for further purification was equilibrated in a TBS buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM MgCl₂, 1 mM MnCl₂, pH 7.5), applied to a column, allowed to stand at room temperature for 3 hours, and washed with 10 times the column volume of a washing buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM MgCl₂, 1 mM MnCl₂, 100 mM octyl glucopyranoside, pH 7.5). After completion of washing, an elution buffer (20 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, 50 mM octyl glucopyranoside, pH 7.5) was used to elute the protein bound to the column. The eluate was collected and dialyzed against PBS(-).

(4) SDS-PAGE

Sub D55 The eluted fraction of (3) was subjected to SDS-PAGE using 7.0% acrylamide gel under non-reducing or under reducing condition, and the gel was stained with Coumassie-blue. As a result, a band considered to be attributable to $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex was observed. Under reducing condition, two bands (185 kDa and 135 kDa) considered to be attributable to integrin $\alpha 2 \cdot \text{IgG}$ heavy chain chimeric protein and integrin $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein were observed. These results suggest that the eluted protein has a molecular structure considered to be $\alpha \alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and is linked by a disulfide bond between the IgG heavy chains.

Example 15

Identification of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and examination of its structural stability

Sub D56 The eluted protein of (3) of Example 14 was ^{125}I -labeled, and subjected to immunoprecipitation using the beads coupled with normal murine IgG, anti-human integrin $\alpha 2$ antibody (clone P1E6) or anti-human integrin $\beta 1$ antibody (clone 4B4) as described in Example 7, and to SDS-PAGE/autoradiography under reducing condition.

Sub D57 As a result, in both 1 mM MgCl_2 and 10 mM EDTA, from the beads of both anti-human integrin $\alpha 2$ antibody and anti-human integrin $\beta 1$ antibody, the same precipitation patterns expected from the structure of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex could be obtained. These results show that the eluted protein obtained in (3) of Example 14 is certainly $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and with the results of (4) of Example 14 also taken into account, it is strongly suggested that the association of both the proteins is stable through a disulfide bond existing the IgG heavy chains.

Example 16

Examination on the capability of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex to be bound

to a collagen, and its specificity

The capability of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex to be bound to a collagen which is a ligand of integrin $\alpha 2 \beta 1$ was examined.

Sup58 > At first, a collagen (Cell Matrix Type1 3 mg/ml) was diluted to 0.1 $\mu\text{g/ml}$ by 0.02M acetic acid solution, and put in an immunoplate by 100 $\mu\text{l/well}$, being kept at 4°C for 16 hours. Then, the collagen solution was removed by suction, and the plate was washed with PBS(-) twice for neutralization. Heat-denaturated 1% BSA-PBS solution was put in the plate by 300 $\mu\text{l/well}$ for blocking at room temperature for 3 hours. After completion of blocking, it was rinsed with PBS(-) twice, to prepare a collagen coated plate.

The cultured supernatant of CHO (100 μl) containing $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex was reacted at 30°C for 3 hours. After completion of reaction, as described in Example 9, the amount of bound $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex was determined.

As a result, as shown in Fig. 4, the absorbance showing the binding of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex to the collagen was increased. The binding was almost perfectly inhibited in the coexistence of 10 $\mu\text{g/ml}$ of anti-human integrin $\alpha 2$ antibody (clone P1E6) and anti-human integrin $\beta 1$ antibody (clone 4B4),

or in the presence of 5 mM EDTA respectively. This result shows that $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex can be bound to a collagen like the integrin $\alpha 2 \beta 1$ existing on the surfaces of cell membranes, and furthermore that the binding is $\alpha 2 \beta 1$ -specific and that the feature of the binding that it depends on cations is retained.

Example 17

Acquisition of a peptide capable of being bound to $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex, and evaluation of its inhibitory activity

At first, $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex purified in Example 6, or human IgG was prepared at a proper concentration by PBS(-) and was coated on a plastic plate at 4°C for 16 hours, being formed as a solid phase on a plastic plate. Then, according to a report (Cott, J. K. and Smoth, G. P., Science, 249, 386-390 (1990)), a phage peptide library in which a random six amino acid residues were cyclized by the disulfide bond of cysteine at both the ends was prepared and suspended in 0.1% BSA-containing TBS buffer. The phage peptide library was reacted with human IgG at 30°C for 3 hours, to absorb phage peptides capable of being bound to IgG. Then, the non-absorbed phases were reacted with $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex at 30°C for 3 hours, and

the reaction mixture was washed with 0.1% BSA-containing TBS buffer twice to remove the phage peptides incapable of being bound to the heterodimer complex. Only the phage peptides capable of being bound were collected after elution with 0.1M glycine-hydrochloric acid (pH 2.2). After collection, the phage was amplified and the above mentioned binding operation was repeated further twice. The only the phage peptides capable of being bound to the heterodimer complex were selectively concentrated. In the final elution operation, phage peptides capable of being bound to the heterodimer complex were eluted using 10 mM EDTA and 0.1M glycine-hydrochloric acid in two steps, and the amino acid sequences of the respective peptides were analyzed. Of them, eight sequences (sequence Nos. 24 to 31) are shown in Table 1. Furthermore, they were examined using the binding assay system of Example 9, and the IC50 values of the four peptide sequences showing binding inhibitory activity are shown in Table 1.

Table 1

Elution condition	Sequence								Inhibitory activity IC50 (μ M)	Sequence No.
EDTA	Cys*	Ile	Pro	Glu	Leu	Ile	Val	Cys*	1.2	24
	Cys*	Met	Arg	Tyr	Thr	Ser	Ala	Cys*	2.3	25
	Cys*	Glu	Trp	Met	Lys	Arg	Phe	Cys*		26
	Cys*	Tyr	Thr	Thr	Arg	Leu	Lys	Cys*		27

Glycine-hydrochloric acid	Cys*	Leu	Arg	Tyr	Ser	Val	Pro	Cys*	1.8	28
	Cys*	Ile	Val	Asn	Arg	Leu	Gly	Cys*		29
	Cys*	Gly	Leu	Gln	Ala	Leu	Pro	Cys*	10	30
	Cys*	Lys	Leu	Lys	Gly	Thr	Met	Cys*		31

Cys* indicates a disulfide bond.

Example 18

Acquisition of a low weight molecular compound capable of inhibiting the binding between the peptide fragment on fibronectin and $\alpha 4 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex

Reagents and reported compounds were picked up at random, adjusted to a final concentration of 50 or 100 $\mu\text{g/ml}$, and added to the binding determination system in Example 9. Compounds showing inhibitory activity were obtained. Of the obtained compounds, the binding inhibitory activities of the four compounds of Norethynodrel (Sigma), D-Penicillamine (Aldrich, Weigert, W. M. et al., Angew. Chem. Int. Ed. Eng., 14, 330-336 (1975), γ -2-Naphthyl butyric acid (Fieser, L. F. J. Am. Chem. Soc., 70, 3197-3203 (1948)), 1-Adamantaneacetic acid (Aldrich) were shown in Table 2.

Table 2

Name of compound	Concentration ($\mu\text{g/ml}$)	Inhibition rate (%)
Norethynodrel	50	28
D-Penicillamine	50	51
γ -2-Naphtyl butyric acid	100	37
1-Adamantaneacetic acid	100	65

Example 19

Preparation of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain
chimeric protein heterodimer complex liposome

A liposome was prepared according to the Martin et al.'s method (Martin, F. J. et al., Biochemistry, 20, 4229, (1981)). At first, an activated SH group was introduced into dipalmitoyl phosphatidyl ethanolamine (DPPE, Sigma) using di-crosslinking reagent N-succineimidyl 3-(2-pyridyldithio)propionate (SDPD, Sigma), to prepare pyridylthiopropionyl dipalmitoyl phosphatidyl ethanolamine (PDP-DPPE). The PDP-DPPE, dipalmitoyl phosphatidyl choline (DPPC) and cholesterol were mixed, to prepare a lipid film, and it was treated by a sonicator. Then, a filter was used to obtain a liposome uniform in diameter (PDP-DPPE liposome). Then, $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex or human IgG (Cappel) used as a negative control were dissolved in a Hepes buffer (100 mM Hepes, 150 mM NaCl, pH 8.0), and SDPD was added for reaction for 30 minutes. The reaction solution was applied to PD-10 column (Pharmacia), and eluted by 0.1M acetic acid-sodium acetate buffer (pH 5.5). To the eluate, dithiothreitol was added for treatment for 20 minutes, and the mixture was applied to PD-10 column again and eluted by a Hepes buffer (100 mM Hepes, 150 mM NaCl, pH 8.0), to obtain SDPD coupled $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain

chimeric protein heterodimer complex. The SDPD modified heterodimer complex and the PDP-DPPE liposome were caused to react with each other at room temperature for 24 hours, and the reaction mixture was separated by Sepharose 4B column (Sigma). From the peak fraction, $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex liposome was obtained.

Sub-Del The amount of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex bound on the liposome was determined by a densitometer (ATT0) after SDS-PAGE/Coumassie staining, and adjusted to final concentration of 1 mg/ml.

Example 20

Flow cytometry of $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex liposome

$\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex liposome was dispersed in 1 mM EDTA-containing PBS(-), and caused to react with anti-human integrin $\alpha 2$ antibody (clone P1E6) or anti-human integrin $\beta 1$ antibody (clone 4B4) at room temperature for 30 minutes.

After completion of reaction, the reaction mixture was centrifuged at 15000 rpm for 10 minutes, being followed by washing with 1 mM EDTA-containing PBS(-) and suspended into the solution again. Into the suspension, FITC labeled anti-murine IgG antibody (Cappel, 10 $\mu\text{g/ml}$) was added as a secondary antibody, and reacted at room temperature for 30

minutes. After completion of reaction, the reaction mixture was similarly washed by centrifugation, and flow cytometry analysis (ELITE, Coulter) was performed.

As a result, the positive reactions for both the antibodies were detected, confirming that $\alpha 2$ ·IgG heavy chain- $\beta 1$ ·IgG heavy chain chimeric protein heterodimer complex was bound on the liposome.

Example 21

Binding activity of $\alpha 2$ ·IgG heavy chain- $\beta 1$ ·IgG heavy chain chimeric protein heterodimer complex to a collagen

Sub D62
A collagen (Cell Matrix Typel, 3 mg/ml) was diluted by 0.02M acetic acid solution, and the solution was put in an immunoplate by 100 μ l/well, being kept at 4°C for 16 hours. Then, the collagen solution was removed by suction, and the plate was washed with PBS(-) twice for neutralization, and heat-denaturated 1% BSA-PBS solution was put in the plate by 300 μ l/well for blocking at room temperature for 3 hours. After completion of blocking, the plate was rinsed with PBS(-) twice, to prepare a collagen coated plate.

Sub D63
Normal human plasma (George King) and von Willebrand's factor deficient (severe) plasma (George Kind) were treated with anti-human IgG antibody and protein A, and dialyzed against PBS(-) for 24 hours, to remove the contained sodium citrate. In order that the Ca ion and Mg ion concentration might be a physiological concentration in the blood when used,

CaCl₂ and MgCl₂ were added to achieve final concentrations of 1.2 mM and 0.2 mM respectively. Into the normal human plasma and von Willebrand's factor deficient plasma adjusted in cation concentration, $\alpha 2$ -IgG heavy chain- $\beta 1$ -IgG heavy chain chimeric protein heterodimer complex liposome or human IgG liposome was suspended to achieve protein concentrations of 1 to 100 ng/ml. Any of the suspensions was put in the collagen coated plate by 100 μ l/well. The plate was shaken by a plate shaker at 100 rpm, for reaction at room temperature for 15 minutes. After completion of reaction, the non-bound liposome was removed by washing with a PB solution (1.2 mM CaCl₂, 0.2 mM MgCl₂, 1% BSA-containing PBS, pH 7.4), and the bound liposome was immobilized by 1% glutaraldehyde-PBS at room temperature for 30 minutes. After completion of immobilization, a heat-denaturated BSA-PBS solution was used for blocking at room temperature for 1 hour. Then, as described in Example 16, it was caused to react with biotin labeled human IgG antibody used as a primary antibody and avidin labeled horseradish peroxidase used as a secondary antibody, and washed with a TBS buffer. Into it, orthophenylenediamine was added as a substrate for color development, and the absorbance at 490 nm were measured. To examine the effect of 5 mM EDTA, anti-integrin $\alpha 2$ antibody (clone P1E6, 10 μ g/ml) and anti-integrin $\beta 1$ antibody (clone 4B4, 10 μ g/ml), it was caused to react with the liposome suspension at room temperature for 15 minutes

Sub D63
cont
Sub D64

before reaction with the collagen.

The results are shown in Figs. 5 and 6. In the normal human plasma, the human IgG liposome as a negative control was not found to be bound to the collagen, but the binding of $\alpha 2 \cdot$ IgG heavy chain- $\beta 1 \cdot$ IgG heavy chain chimeric protein heterodimer complex liposome to the collagen was increased with the concentration dependent on manner. Also when the von Willebrand's factor deficient plasma was used, equivalent binding was detected. Furthermore, the binding to the collagen observed when 30 ng/ml of $\alpha 2 \cdot$ IgG heavy chain- $\beta 1 \cdot$ IgG heavy chain chimeric protein heterodimer complex liposome was added to the normal plasma was completely inhibited by adding EDTA as a cation chelating agent or the antibodies. The results show that in plasma with a physiological cation concentration, $\alpha 2 \cdot$ IgG heavy chain- $\beta 1 \cdot$ IgG heavy chain chimeric protein heterodimer complex liposome is bound to a collagen like platelets, and strongly suggest that it can be a substitute of adhesive platelets, and can be a reagent for monitoring the collagen exposed region. Furthermore, it is indicated that since equivalent binding activity was shown also in von Willebrand's factor deficient plasma, the liposome can also be used in the plasma with coagulation abnormality such as von Willebrand's disease.

Example 22

Analysis of collagen covering state by $\alpha 2 \cdot$ IgG heavy chain- β

1 · IgG heavy chain chimeric protein heterodimer complex
liposome

Five microliters of a collagen solution was spotted at the center of each of the wells of a Lab-Tek chamber slide (Intermed, 8-well type, plastic) and allowed to stand for 16 hours, then washed and treated for blocking. Then, a suspension in which $\alpha 2 \cdot$ IgG heavy chain- $\beta 1 \cdot$ IgG heavy chain chimeric protein heterodimer complex liposome was suspended in normal human plasma to achieve a protein concentration of 30 ng/ml as described in Example 21 was put in the slide by 200 μ l/well, for reaction under the same conditions. After completion of reaction, the non-bound liposome was removed by washing with a PB buffer, and the retained was immobilized and treated for blocking. Then, it was bound to biotin labeled anti-human IgG antibody as a primary antibody and with avidin labeled horseradish peroxidase as a secondary antibody, and was washed with a TBS buffer. After completion of washing, diaminobenzidine was added for staining, to observe the covering state of the $\alpha 2 \cdot$ IgG heavy chain- $\beta 1 \cdot$ IgG heavy chain chimeric protein heterodimer complex liposome bound on the collagen.

With the human IgG liposome, the collagen coated portion was not stained, but with $\alpha 2 \cdot$ IgG heavy chain- $\beta 1 \cdot$ IgG heavy chain chimeric protein heterodimer complex liposome, the collagen coated portion was entirely stained. Therefore,

since the $\alpha 2 \cdot \text{IgG}$ heavy chain- $\beta 1 \cdot \text{IgG}$ heavy chain chimeric protein heterodimer complex liposome covered the collagen coated portion only, it was strongly suggested that the liposome could be a substitute of adhesive platelets.

Industrial Availability:

The present invention provides integrin-immunoglobulin chimeric protein heterodimer complexes in which the α chain and the β chain of an integrin are stably associated. The obtained integrin-immunoglobulin chimeric protein heterodimer complexes can be directly used as drugs, and can also be used for determining the binding between an integrin and a ligand, and searching for a substance capable of being bound to an integrin and a substance inhibiting the binding between an integrin and a ligand. They can also be used as diagnostic reagents.

Furthermore, among the heterodimer complexes, especially integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex can be used as a substitute of platelets. Furthermore, integrin $\alpha 2 \beta 1$ -immunoglobulin chimeric protein heterodimer complex can be used as a therapeutic or preventive agent for bleeding tendency involved in thrombocytopenia, platelet function abnormality, etc. Furthermore, it can also be used as a reagent for monitoring the exposed region of an extracellular matrix and for the targeting therapy.